CHARACTERIZATION OF THE CHEMICAL DEFENSES OF *SAGITTARIA GRAMINEA*, A FRESHWATER PLANT, AGAINST CRAYFISH HERBIVORY

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ABSTRACT

Herbivores powerfully impact community structure by altering biomass, species richness, and succession, which cause many plants to evolve chemical defenses against them. Chemical defenses have been well studied in marine and terrestrial systems, but studies focusing on freshwater chemical ecology have only recently begun to increase in number. The freshwater macrophyte *Sagittaria graminea* is relatively nutritious and found in many of the same habitat types as crayfish, which can consume large amounts of biomass. We looked to chemical defenses to explain how *S. graminea* can maintain its populations despite the presence of crayfish. In this study, we aimed to characterize the deterrent secondary metabolite(s) found in *S. graminea* through bioassay-guided fractionation. Additionally, we compared deterrence of stem and leaf regions to test for differential allocation of resources within individuals. Significant deterrence by several distinct fractions of *S. graminea* extracts indicates that more than one chemical is contributing to *S. graminea*'s defenses. Additionally, one of these fractions contains chemical characteristics similar to other identified freshwater plant defensive chemicals. We determined that the deterrent chemicals have relatively low polarity. However, the lack of significant differences between consumption of the stem and leaf region of *S. graminea* indicates that defenses are equally allocated throughout the individuals, despite the easier accessibility of the stem to crayfish in the wild. Characterization of the deterrent chemical(s) and investigation of differential resource allocation contribute to a relatively unexplored area of chemical ecology by providing insight into the structure and regulation of a widely distributed macrophyte's defense.

Key words: *Sagittaria graminea*, *Procambarus clarkii*, chemical defense, optimal defense theory

INTRODUCTION

Freshwater Ecosystems

Freshwater plants, also known as macrophytes, are arguably some of the most influential organisms in rivers, lakes, and streams. They provide habitats, food, and stabilization of abiotic factors. For example, the removal of macrophytes can result in decreased flow velocity in a stream or river. This reduced flow makes the habitat unsuitable for a significant number of macroinvertebrates, which causes the community to decrease in density and diversity (Milisa et al. 2006). Even a single macrophyte species can have strong impacts on its habitat. For instance, millfoil (*Myriophyllum sibiricum*) can be responsible for a 70-1725% increase in abundance of major invertebrate taxa in a Colorado reservoir (Cronin et al. 2006). Millfoil presence increases the abundance of organisms not because it provides food, but because it provides habitat and surfaces for biofilms to colonize (Cronin et al. 2006). These biofilms lead to the production of periphyton, an important food source for invertebrates. Understanding the complex effects of macrophytes will provide insight into ecosystems on which humans depend.

These ecosystems, however, are subject to major impacts by herbivores. The effects of herbivores in freshwater environments can be far-reaching and intense. For instance, the rodent nutria (*Myocastor coypus*) can significantly reduce the above-ground biomass of coastal marsh plants in its habitat (Taylor and Grace 1995). Similarly, multiple herbivorous snail species can decrease species richness and biomass of submerged macrophyte communities (Sheldon 1987). Herbivores commonly affect interactions among plants by means such as altering the rate of succession, shifting succession onto a new path, increasing or decreasing competition, and facilitating mutualism (Hixon and Brostoff 1996, Taylor et al. 1997).

The primary reason herbivores are able to direct such significant changes in plant communities is that they are selective. For example, one study demonstrated that snails (*Potamopyrgus antipodarum*) altered the composition of controlled microalgal communities by preferentially consuming erect diatoms instead of basal cells. This preference not only affected the species richness in the community, but also had a positive effect on diversity when the community was allowed to recover (Holomuzki et al. 2006). Herbivory can become complicated when considering different circumstances, but they generally prefer plants that take a short time to process, are rich in nutrients, and do not contain toxic or unpalatable compounds (Holdich 2002). Because plant defenses alter herbivore consumption, they can affect the entire community.

Study Organisms

The model herbivores in the present study will be crayfish, freshwater crustaceans that generally cause large direct effects and complex indirect effects through plant consumption (Creed 1994, Lodge et al. 1994, Nystrom et al. 1996, Dorn and Wojdak 2004). In one Michigan stream, the presence of crayfish (*Orconectes propinquus*) decreased the biomass of filamentous algae *(Cladophora glomerata)* by ten times in comparison to a non-crayfish-inhabited section of the stream (Creed 1994). Another study found that lakes with higher crayfish (*Pacifastacus leniusculus* Dana) abundance have lower macrophyte coverage, abundance, and species richness (Nystrom et al. 1996). Greater crayfish densities complicate food webs, increase bioturbation, and prevent vascular plant establishment (Lodge et al. 1994, Dorn and Wojdak 2004). The present study will use the crayfish species *Procambarus clarkii*. Although native to the southeastern United States, and thus not considered an invasive species in the context of this study, *P. clarkii* can have major impacts when introduced into a new habitat, based on its ability

to tolerate environmental changes and to consume a variety of resources (Gutierrez-Yurrita and Montes 1998, Kreider and Watts 1998). Therefore, this species is more likely to respond to laboratory tests in a natural manner than some other types of grazers. Overall, crayfish are manageable invertebrates to use in laboratory studies, but are also highly relevant to freshwater ecological studies.

This study aims to understand how a specific macrophyte, *Sagittaria graminea*, defends itself against crayfish herbivory. A previous study featured *S. graminea* as one of fourteen freshwater macrophytes from the Southeastern U.S. and quantified its nutritional content (Cronin et al. 2002). While *S. graminea* is moderately nutritious compared to the other fourteen species surveyed, its consumption by the crayfish *P. clarkii* was relatively low. However, the amount of plant tissue consumed was not directly compared to a control, so it is difficult to determine how well defended this species is (Cronin et al. 2002). Prusak et al. (2005) also explored the chemical defenses of various freshwater plants. In their study, the organic extract of *Sagittaria latifolia*, mixed into an artificial diet, significantly reduced feeding by *Procambarus acutus*, indicating the presence of a chemical defense (Prusak et al. 2005). Therefore, it is likely that *S. graminea* has evolved a chemical defense to help decrease the negative impacts of crayfish herbivory.

Chemical Defenses

Considering the challenges predators and herbivores present to their prey's survival, the evolution of defenses seems logical. This is particularly relevant to freshwater systems where herbivores consume about three times more annual primary production than in terrestrial systems (Cyr and Pace 1993). Chemical defense research in freshwater habitats is not as active as research in marine and terrestrial systems because it was previously assumed that herbivory was not influential in this system, but it is gaining interest and needs more studies to proceed (Burks

and Lodge 2002). Research has uncovered a variety of defensive strategies that include prey chemical defenses causing lost appendages, inducing vomiting, and reducing reproductive success in marine predators (Lindquist and Hay 1995, Pohnert 2005, Kicklighter and Hay 2006). A few studies have shown promise for highly evolved and diverse chemical defensive strategies in freshwater plants as well (Cronin et al. 2002, Rowell and Blinn 2003, Prusak et al. 2005, Parker et al. 2006, Miller and Provenza 2007, Parker et al. 2007). A highly evolved defense can sometimes be observed when it is exploited by another species. For example, the chemically defended aquatic moss *Fontinalis novae-angliae* serves as a refuge for small, sedentary herbivores against larger generalist grazers (Parker et al. 2007). The amphipod *Hyalella azteca* frequently consumes the chemically defended roots of *Berula erecta*, which decreases the rate that it is preyed upon (Rowell and Blinn 2003). The presence of diverse chemical defensive strategies and the dependence on these strategies across trophic levels indicate that freshwater habitats may offer as many insights about chemical communication as marine and terrestrial habitats.

Identification of deterrent chemicals is particularly advantageous to advancing freshwater chemical ecology. With this knowledge, the secondary metabolite production of plants across habitats can be compared and contrasted. Additionally, commonly used chemicals such as caffeine and nicotine originally evolved as defensive secondary metabolites in plants. Despite the benefits to exploring these chemicals, only a handful of studies have demonstrated chemical defenses of freshwater plants and isolated the chemicals responsible (Newman et al. 1996, Bolser et al. 1998, Wilson et al. 1999, Kubanek et al. 2001, Parker et al. 2006, Erhard et al. 2007, Parker et al. 2007). Watercress (*Nasturtium officinale*) allocates hydrolyzed glucosinolates to its leaves when they are fresh and high in nitrogen content (Newman et al. 1996). The novel metabolite

habenariol was identified in orchids (*Habenaria repens*) as a deterrent to crayfish (*Procambarus clarkii*) feed (Bolser et al. 1998, Wilson et al. 1999). Kubanek et al. (2001) and Parker et al. (2006) identified lignoids as the deterrent chemicals in *Saururus cernuus* and *Micranthemum umbrosum*, respectively. Previously unknown flavonoids were identified as deterrent compounds in *Elodea nuttallii* against the generalist aquatic moth, *Acentria ephemerella* (Erhard et al. 2007). Additionally, a C_{18} acetylenic fatty acid was found to be responsible for the deterrence of Canada geese (*Branta canadensis*) and crayfish (*Procambarus spiculifer)* from aquatic moss (*Fontinalis novae-angliae*) (Parker et al. 2007). Based on these few examples, we can make the conclusion that freshwater plants have the ability to produce a variety of chemical defenses. Two commonly identified types of chemical defenses, specifically alkaloids and phenolics, are represented by the glucosinolates, habenariols, and lignoids (Rockwood 2006). Fatty acids are less commonly found to be defensive chemicals in plants. The details of these studies allow us to see trends in chemical defense and offer opportunities for us to explore further uses of these chemicals.

Generally, deterrent chemicals can be present in multiple contexts: as a single compound, as a mixture of several compounds, or as higher concentrations of normally non-deterrent compounds. It is difficult to assess how *S. graminea* will be chemically defended because no previous studies have identified chemical defenses of the genus *Sagittaria* or any other genus in the family *Alismataceae*. Based on past findings of other freshwater plants (Bolser et al. 1998, Kubanek et al. 2001) and preliminary data on this species (Sieg and Rasher unpublished data), we hypothesized that the deterrent chemical(s) in *S. graminea* have low polarity. However, prior to the initiation of this study there were not enough data to predict the structure or structural class of the deterrent chemical or whether multiple compounds are involved.

Differential Defense Allocation

Crayfish are bottom-dwellers, suggesting that the part of *S. graminea* most susceptible to crayfish herbivory by *P. clarkii* should be the stem. The optimal defense theory predicts that the most vulnerable or valuable parts of the plant are most likely to be highly defended. This often applies to parts of the plant that are particularly nutritious, structurally important, or in the early stages of development (Cronin 2001). Optimal defense theory has been positively demonstrated in a number of marine and terrestrial organisms, but application of this theory to freshwater plants is limited (Ohnmeiss and Baldwin 2000, Schupp et al. 1999, Wackers and Bonifay 2004, Zangerl and Rutledge 1996). Chemical defenses may be in the form of constitutive defenses or inducible defenses. Constitutive defenses are consistently present in the plant while inducible defenses are produced only when the prey senses a cue from the predator, which may be by sight (for animal prey), chemical cue, or physical damage (Cronin 2001). We are interested in focusing on constitutive defenses for the purpose of evaluating optimal defense theory because Prusak et al. (2005) found that *Sagittaria latifolia* did not contain inducible defenses, so it is likely that *S. graminea* does not contain them either. Due to the accessibility and structural importance of stems, we hypothesize that this part of *S. graminea* individuals will have stronger constitutive chemical defenses than the leaves.

Overview

This study characterized the chemical compounds responsible for *S. graminea's* defense against the common freshwater herbivores, *P. clarkii*. We used behavioral feeding assays with crayfish to narrow down the traits of the compounds based on the crayfishes' preferences of extracts compared to controls. Various techniques including liquid-liquid extraction and column chromatography were used to fractionate *S. graminea* extractions. Additionally, we explored

differences in stem and leave constitutive defenses through fresh tissue and crude extract bioassays. The findings of this study will contribute to the growing field of freshwater chemical ecology and help us better understand herbivore-macrophyte interactions.

METHODS

Organisms and Bioassays

Sagittaria graminea, or the grassy arrowhead, is a freshwater angiosperm with emergent leaves (Stuzenbaker 1999). It is found in a variety of habitats, including ponds, small streams, drainage ditches, and marshy shores (Godfrey and Wooten 1979). Twenty of these plants were collected in the fall of 2007 and four more were collected in the fall of 2010 from Clayton County Water Authority in Hampton, Georgia. This species was selected because it is easy to collect and it is comparably nutritious due to moderate nitrogen and phosphorus content (Cronin et al. 2002). A 150.09 g fresh sub-sample of this collection was blotted dry and the wet mass was recorded. The sub-sample, which consists of stems, was then freeze-dried and stored at -80 ^{o}C. The second sample collected in the fall of 2010 for the differential resource allocation analysis and continuation of the chemical defense characterization was treated in the same way.

The herbivore that we chose to test the chemical defenses of *S. graminea* is the omnivorous crayfish *Procambarus clarkii*. All bioassays except those testing the size exclusion elucidated sub-fractions were conducted with only *P. clarkii*. Crayfish were chosen as the herbivore species because they are easy to obtain and are generalist feeders. Fifty small crayfish were ordered from the Carolina Biological Supply Company in the spring of 2010 and were replenished twice during the fall of 2010 and the spring of 2011. All crayfish were kept in separate compartments of a 2 L plastic tub with re-circulating fresh water and each have a 10 cm section of PVC pipe to use as shelter.

The crayfish were fed every two or three days with about 2 g of BioBlend herbivore food. They were fed plates containing 60 indentations that were half-filled with a 1:1 mixture of finely

ground freeze-dried broccoli and lettuce (hereafter, "broclet") several times periodically prior to the bioassay in order to familiarize them with the set-up. Broclet was solidified with 10 mL of deionized water and 0.19 g agar for every 1 g of broclet. Throughout the experiment, chemical extracts of *S. graminea* were dissolved in methanol and mixed together with broclet. The amount of extract added to the broclet was calculated using the natural concentration of that chemical extract fraction found in 22 g of freeze-dried *S. graminea*. This ratio was corrected for the amount of extract that was lost to previous bioassays and the fractionation process. The mass of the extract to be added was subtracted from the mass of the broclet. The two components, weighing about 1.4 g total (14 ml of food multiplied by plant density), were combined in a 20 ml scintillation vial and dried with a speed vacuum. An equivalent volume of methanol that was added to the experimental broclet was also added to the control broclet, and this was dried with the speed vacuum. De-ionized (DI) water was added to the dried broclet (3.5 ml/1 g broclet treatment) and the food was solidified with boiling agar (0.19 g agar and 6.5 ml DI water/1 g broclet treatment). The experimental and control foods were spread onto separate, labeled sides of the feeding plates with plastic spatulas.

Other than variations related to the extract used in the treatment, all of the feeding assays were conducted in the following manner. Fifteen to twenty-five replicate feeding plates were prepared for each bioassay. The plates were covered and refrigerated if the bioassay could not be performed immediately, but all bioassays were performed within two hours of plate preparation. If crayfish were in their shelters, they were removed and allowed to settle for two minutes. Shelters were removed so as not to block the feeding plates. The plates were placed in each compartment facing the same direction to remove effects of water flow and were alternated with the control on the left or right. Plates were removed from the crayfish when 50-90% of the

broclet-filled indentations on the plate had been consumed. When possible, we waited until most or all of one side had been consumed in order to identify a preference. Bioassays were performed within a single day, only one was performed each day, and they lasted two to five hours each, depending on how quickly the crayfish ate at least half of their plates. The number of indentations consumed from each side of the plate was counted and recorded. A two-tailed paired t-test was performed to determine if a significant different between the means existed.

Deterrent Chemical Isolation

The first step in isolating the deterrent compound was to determine whether *S. graminea* is deterrent at all by testing its crude extract. The volumetric displacement of 22 g of freeze-dried S. *graminea* stems was measured in methanol. Keeping it submerged in the methanol, it was then homogenized and 600 ml of dichloromethane was added to create a 1:1 mixture. The plant tissue was kept in the extraction mixture for one hour. The solvents were removed with vacuum filtration and dried with a rotary evaporator. The plant tissue was then transferred to two more rounds of extraction solvents, each lasting one hour. The second and third rounds of extraction were each completed with 600 ml of 2:1 dichloromethane/methanol, which were dried in the round-bottom flask containing the previously dried extraction solvents. A bioassay, as described above, was used to test the deterrence of the crude extract. Percent deterrence was calculated by subtracting the average number of treatment indentations consumed from the average number of control indentations consumed, dividing this difference by the average number of control indentations consumed, and multiplying the quotient by 100.

Once deterrence by the crude extract was confirmed, liquid-liquid extraction was used to further fractionate the crude extract. The first step of liquid-liquid extraction was dissolving the crude extract in methanol and depositing the volume of methanol containing 3.66 g of extract

into a separatory funnel. Methanol and water were added to the separatory funnel to create a ratio of 9:1, respectively, with a total volume of 300 ml. This mixture represented the more polar phase of the first round of fractionation. The non-polar phase was 300 ml of hexanes. The solvents and crude extract were shaken in the funnel several times and a distinct layer between the two phases formed when the funnel was placed in a clamp. The hexanes layer, the top layer, was removed from the funnel and dried with a rotary evaporator. The methanol/water layer was diluted to 6:4 methanol/water, with a total volume of 450 ml. An additional 450 ml of chloroform was added to the funnel to serve as the less polar phase of the second round of extraction. The process of shaking was repeated and the two phases were dried separately on the rotary evaporator. Normal phase silica thin layer chromatography (TLC) was used to ensure that the three fractions produced from liquid-liquid extraction contained distinct compounds. The plate was first developed with UV radiation, then with sulfuric acid and heat. One bioassay was performed for each of the three fractions.

The fractions that were rejected by the crayfish were further fractionated with column chromatography. A normal phase silica column (length of 7.5 cm, diameter of 2.7 cm) and five mobile phases were used to separate 0.09 g of the hexanes-soluble extract fraction and 0.25 g of the chloroform-soluble portion. The mobile phases for the hexanes-soluble portion were 50 ml 100% hexanes, 50 ml 85:15 hexanes/ethyl acetate, 50 ml 70:30 hexanes/ethyl acetate, 75 ml 50:50 hexanes/ethyl acetate, and 100 ml 100% ethyl acetate. The mobile phases for the chloroform-soluble portion were 50 ml 100% hexanes, 50 ml 80:20 hexanes/ethyl acetate, 50 ml 50:50 hexanes/ethyl acetate, 75 ml 20:80 hexanes/ethyl acetate, 250 ml 20:80 methanol/ethyl acetate, and 50 ml acetonitrile. The same methods for TLC previously discussed were used to

assess how the fractions produced for each separation should be combined. Bioassays were performed for five hexanes-soluble sub-fractions and seven chloroform-soluble sub-fractions.

A lack of deterrence in any of the hexanes-soluble sub-fractions led to a bioassay using an extract consisting of all the sub-fractions. When it was confirmed that the deterrent chemicals had not lost activity, bioassays of the five hexanes-soluble sub-fractions were repeated. At this point in the project, there was not enough extract left to continue with bioassays and fractionation. Therefore, the second sample of *S. graminea* was extracted and separated with liquid-liquid extraction. The hexanes-soluble and chloroform-soluble fractions were combined and further separated with size exclusion column chromatography. A lipophilic Sephadex LH20 column (length of 80 cm, diameter of 1.5 cm) was used with a mobile phase made up of 10:2.5:1 ethyl acetate/methanol/water. The column separated the molecules within the extract by size, with the larger molecules eluting off the column first. Although further isolation of the deterrent compound(s) was not achieved in this study, solid phase extraction and high pressure liquid chromatography (HPLC) would have been used to further separate the extract. If pure compounds had been isolated, they would have been identified with various methods including ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data, mass spectrometry, infrared spectra, UV measurements, and optical rotation measurements.

Differential Defense Allocation

The *S. graminea* harvested from Clayton County Water Authority in the fall of 2010 was used for this portion of the experiment. Fresh tissue from the stem and leaf of the same plant was cut into small pieces and the blotted wet weight was recorded. This was repeated for four different plants. The paired samples were placed in individual crayfish containers for approximately two hours. Three control pairs were placed in the same tank, but out of reach from

crayfish. The paired tissue samples were removed after the allotted time period. However, if the crayfish was close to consuming the majority of both samples, they were removed at that time. The blotted wet weights of the samples and controls were measured following the assay. Observations determined if experimental samples were never touched by crayfish. This was the case for three leaf samples, which were then used, along with the controls, to calculate the amount of mass lost or gained due to abiotic conditions. The mass change due to abiotic conditions was calculated in two stages of drying following removal: semi-dry and completely dry. This distinction was made because some of the experimental samples dried out quickly while others retained water for an extended period of time, requiring two different conversion factors. The average mass percent loss due to abiotic factors of the corresponding plant part was applied to each sample following the experiment. The mass lost was subtracted from the post-experimental sample weight and the percent consumed was calculated based on this value and the weight of the sample prior to the experiment. A paired two-tailed t-test was used to calculate whether a significant difference existed between the percent consumed of stems and leaves. A second bioassay was conducted, which compared the deterrence of the stem crude extract to the leaf crude extract, each mixed with control broclet. This comparison was performed in the same manner as previous extract assays.

RESULTS

Deterrent chemical isolation

Bioassay-guided fractionation of *Sagittaria graminea* extract has resulted in a multitude of possible explanations for chemical defense. First, the crude extract was found to be deterrent, with extract-containing foods 32% less palatable than controls (p=0.0005) (Figure 1). Following liquid-liquid extraction, two of the three fractions were found to be deterrent. The deterrent fractions were the hexanes-soluble fraction and the chloroform-soluble fraction while the methanol/water-soluble fraction was not (p=0.0033, p=0.016, p=0.43, respectively) (Figure 2). Both the hexanes-soluble fraction and chloroform-soluble fraction resulted in a 23% reduction in feeding compared to controls, making them equally potent (Figure 2a, 2b).

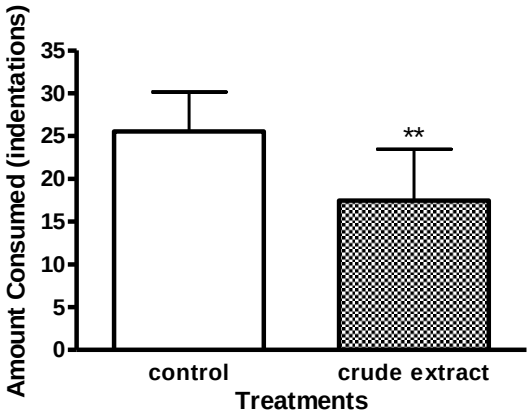

Figure 1. Crude Extract Bioassay. Deterrence of artificial food containing *S. graminea* crude extract toward the crayfish *P. clarkii* (n=11). Total amount possible to consume per treatment is 30. ** indicates p<0.01, as calculated by two-tailed paired t-test.

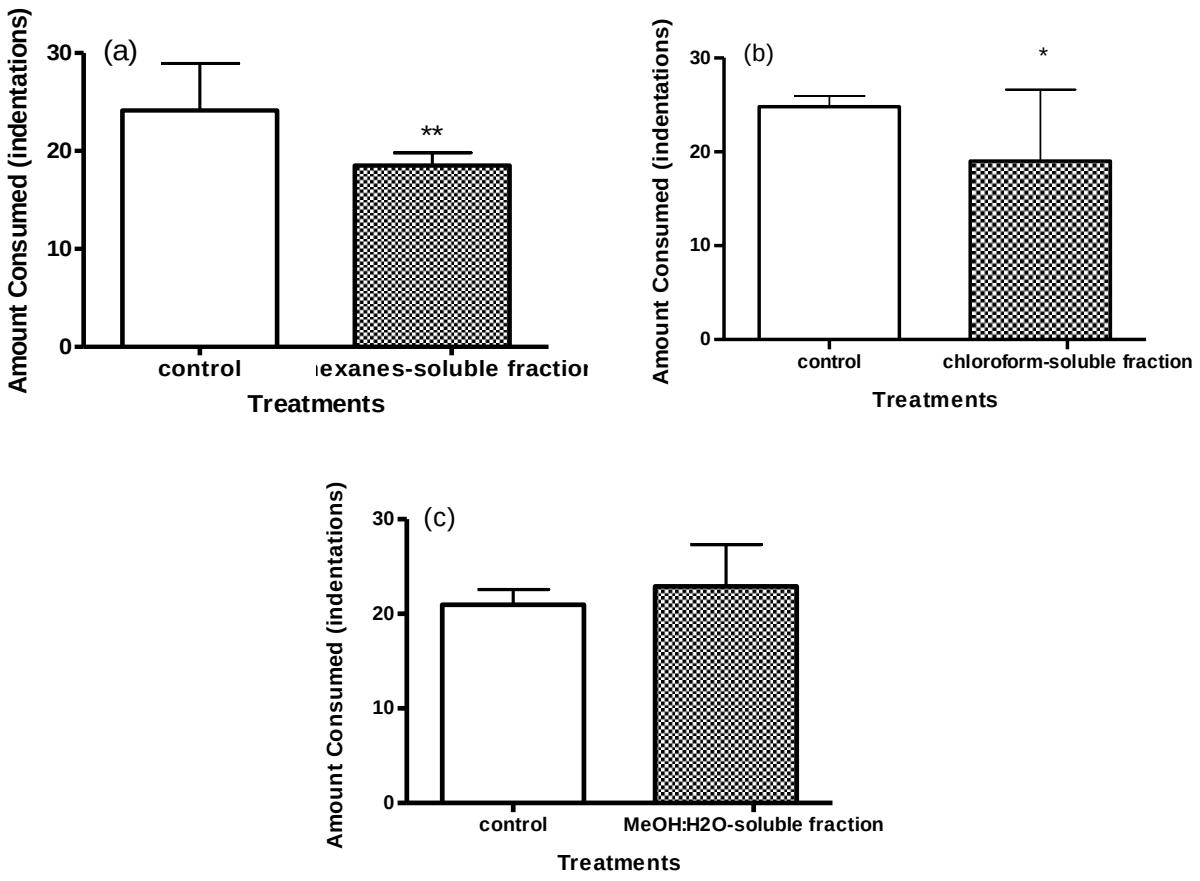

Figure 2. Bioassays for Liquid-liquid Extraction Fractions. The deterrence of artificial food containing the (A) hexanes-soluble fraction (n=16), (B) chloroform-soluble fraction (n=18), and (C) MeOH:H_20-soluble fraction (n=18) of *S. graminea* extract towards the crayfish *P. clarkii*. Total amount possible to consume per treatment is 30. * indicates $p<0.05$, ** indicates $p<0.01$ as calculated by paired T-test.

The hexanes-soluble and chloroform-soluble fractions were each fractioned via silica gel column chromatography. The sub-fractions differ by the polarity of solvents needed to elute them from the column. Sub-fraction 1 was removed with non-polar solvents and each additional sub-fraction was eluted with an increasingly polar solvent mixture. The hexanes-soluble fraction produced five distinct sub-fractions. No deterrent activity was found through the first round of bioassays, so a portion of each sub-fraction was used to recombine all five fractions at their

natural ratios. A bioassay of this recombination extract proved that the extract still contained defensive properties with 41% deterrence (p=0.0380) (Figure 3). Therefore, the sub-fractions were each tested for deterrence again with a second round of bioassays and one of the sub-fractions (4) was found to cause 28% deterrence (p=0.041) (Table 1). Additionally, one fraction was found to be preferred over the control during the first round of bioassays, but this result was not repeatable during the second round.

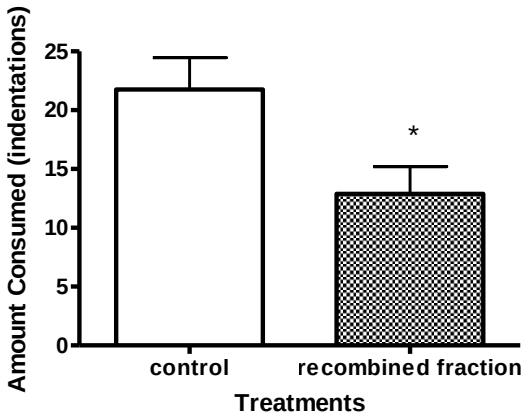

Figure 3. Bioassay for Hexanes-soluble Recombined Fraction. The deterrence of artificial food containing the recombined hexanes-soluble fraction of *S. graminea* toward the crayfish *P. clarkii*, amount consumed out of 30 (n=8). * indicates $p<0.05$, as calculated by paired t-test.

Table 1. Hexanes-soluble Sub-fractions Bioassays. Amount of food containing hexanes-soluble fraction or control that was consumed (out of 30 each) in a paired bioassay (second round bioassays). SEM is standard error of the mean. p-values from paired two-tailed t-test.

Hexanes-soluble sub-fraction	Fraction mean ± SEM	Control mean ± SEM	p-value	n
1	18± 3	16 ± 2	0.57	11
2	17± 3	17 ± 3	0.90	9
3	20 ± 2	19 ± 3	0.72	11

4	18 ± 3	25 ± 1	0.041	11
5	22 ± 2	19 ± 2	0.16	12

The chloroform-soluble fraction produced seven distinct sub-fractions. Four of these seven fractions were found to be deterrent when compared to the control (Table 2). Sub-fraction 1 was eluted off the column with non-polar solvents, and sub-fraction 7 with polar solvents.

Table 2. Chloroform-soluble Sub-fractions Bioassays. Amount of food containing hexanes-soluble fraction that was consumed (maximum is 30) along with control food consumed (maximum is 30) in paired bioassay. SEM is standard error of the mean. p-value from paired two-tailed T-test.

Chlorofom-soluble sub-fraction	Fraction mean ± SEM	Control mean ± SEM	p-value	n	Percent deterrence
1	16 ± 2	21 ± 1	0.048	13	24%
2	13 ± 2	19 ± 2	0.062	14	
3	12 ± 1	21 ± 2	0.0017	16	43%
4	12 ± 2	23 ± 2	0.0002	13	48%
5	13 ± 2	17 ± 2	0.17	14	
6	12 ± 2	18 ± 2	0.022	13	33%
7	19 ± 2	18 ± 2	0.77	14	

Due to the small amounts of sub-fractions remaining after the bioassays described above, a new set of freeze-dried *S. graminea* tissue, collected in 2010, was extracted. TLC analysis of the chloroform-soluble, hexanes-soluble, and methanol/water-soluble fractions demonstrated extreme similarity between the chloroform- and hexanes-soluble fractions. Therefore, these two fractions were combined. This decision is supported by the sub-fraction results because hexanes

sub-fraction 4 showed similar TLC characteristics to chloroform sub-fraction 3, which were both deterrent and came off the silica column with similar ratios of hexanes to ethyl acetate (50:50). Based on these similarities, it is likely these two sub-fractions represent the same compound. Prior to recombination, the chloroform- and hexanes-soluble fractions each tested positive for deterrence against *P. clarkii* (p=0.0002, p=0.0113, respectively).

The now recombined *S. graminea* extract was then separated into sub-fractions with a lipophilic LH-20 Sephadex column. This separation technique resulted in nine separate fractions, grouped by similar TLC characteristics. This type of column separates molecules by size exclusion, which results in large molecules eluting from the column prior to small molecules. Therefore, lower numbered sub-fractions contain larger molecules than higher numbered sub-fractions. Sub-fractions 3 through 7 were tested for deterrence against crayfish first due to intense TLC spots within these fractions compared to the surrounding sub-fractions. It was at this point in the study that a second species of crayfish was added to the bioassays in order to supplement the available number of *P. clarkii*. Sub-fraction 3 was preferred over the control, sub-fractions 4-6 were significantly deterrent compared to the control, and sub-fraction 7 was equally preferred compared to the control (Table 3).

Table 3. Size Exclusion Elucidated Sub-fractions Bioassays. Amount of food containing sub-fractions 3 through 7 that was consumed along with control food consumed (out of 30 for each) in paired bioassay. SEM is standard error of the mean. p-value from paired two-tailed t-test.

Sub-fraction	Fraction mean ± SEM	Control mean ± SEM	p-value	n	Percent deterrence
3	24 ± 1	19 ± 2	0.0339	17	
4	17 ± 2	22 ± 1	0.0396	23	21

5	17 ± 2	23 ± 1	0.0133	19	25
6	13 ± 2	18 ± 2	0.0167	18	28
7	15 ± 2	18 ± 2	0.3461	20	

Differential Defense Allocation

The first bioassay to test differential chemical defense allocation to the stem and leaves used fresh tissue. The average mass percent loss due to abiotic factors was 48% for semi-dry and 86% for completely dry leaf samples. The values for stem samples were 11% for semi-dry and 81% for completely dry. The experimental samples were corrected with the corresponding mass percent loss. There was no significant difference between the consumption of the stem and leaf ($p = 0.80$) (Figure 4a). However, observations determined that the stem portion was more difficult for the crayfish to eat due to the buoyancy and bulkiness of the structure, both of which the leaf lacks. The crude extract bioassay was carried out to eliminate the effects of structural defense and confirm or refute the results found in the fresh tissue bioassay. The bioassay results yielded no significant difference between the deterrence capabilities of the leaf portion and the stem portion ($p = 0.32$), indicating that leaves and stems are similarly palatable and contain similar levels of chemical defenses (Figure 4b).

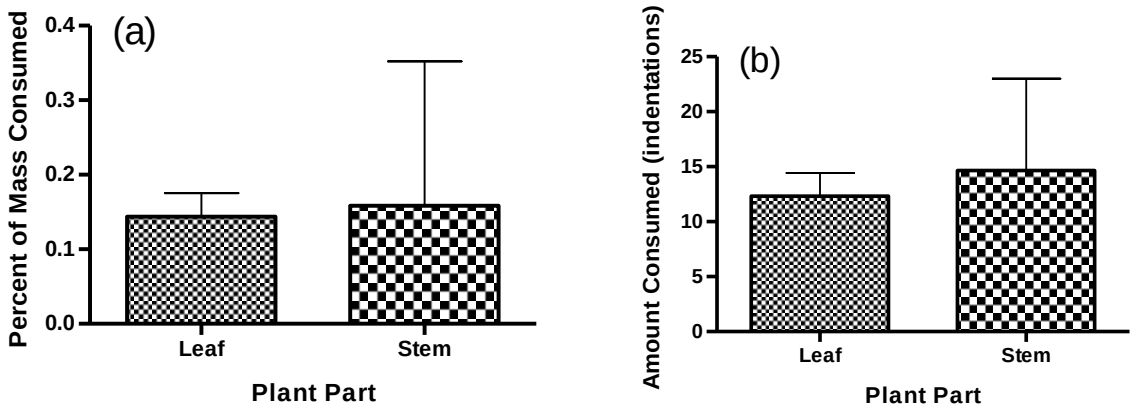

Figure 4. Bioassays for Stem and Leaf Comparisons. (a) Percent of leaf or stem fresh tissue of *S. graminea* consumed and (b) amount of broclet containing leaf and stem crude extract of *S. graminea* consumed by the crayfish *P. clarkii*. Neither comparison showed significant differences.

DISCUSSION

Confirming the presence of *S. graminea*'s chemical defense was expected, yet necessary (Figure 1) (Cronin et al. 2002). Our main hypothesis was that *S. graminea* would possess a chemical defense and that it would have low polarity based on previous research on freshwater plants (Bolser et al. 1998, Kubanek et al. 2001, Prusak et al. 2005). Because the defensive chemicals dissolved in the non-polar hexanes layer (polarity index of 0.0) and the chloroform layer (polarity index of 4.1) during liquid-liquid extraction, our hypothesis was supported (Figure 2). However, some deterrent sub-fractions from the chloroform-soluble layer were attracted to the more polar solvents, which came off the chromatography column towards the end, and are indicated by a higher fraction numbers (Table 2). Therefore, the total chemical of *S. graminea* defense spans a range of relatively low polarities.

Because multiple fractions were found to elicit deterrence from *P. clarkii*, and they all displayed distinctive thin-layer chromatography (TLC) characteristics, it is likely that *S. graminea* has several compounds contributing to its chemical defense. This would not be unique, considering the freshwater macrophyte *Micranthemum umbrosum* has four identified chemicals actively defending it against herbivory (Parker et al. 2006). On the other hand, the deterrent chemicals may not produce UV-visible or sulfuric acid-staining TLC spots, in which case, there may only be one or two chemicals responsible for deterrence. Because the chloroform-soluble fraction and hexanes-soluble fraction caused equivalent decrease in consumption compared to controls, it is also likely that these fractions contain overlapping deterrent compounds (Figure 2). The chloroform-soluble sub-fraction 3 and hexanes-soluble sub-fraction 4 may be the same compound based on similarities in TLC spotting and the ethyl acetate/hexanes ratio that eluted

them from the silica gel columns. This evidence supports the decision to combine the hexanes- and chloroform-soluble fractions during the second round of fractionation because the hexanes-soluble fraction probably does not contain a unique compound

The visible TLC spots are useful for comparing *S. graminea*'s deterrent compounds to those of other freshwater plants. A few spots in sub-fraction one of the chloroform-soluble fraction were visualized with UV light, which indicates the presence of compounds with a conjugated double bond system. TLC spots in the other three sub-fractions of the chloroform-soluble portion were visualized with sulfuric acid and heat, which is used to detect the presence of involatile organic compounds that are readily oxidizable with sulfuric acid in air. The UV light used to develop the TLC plates was set to 254 nm, which is the UV wavelength at which benzene absorbs maximum UV radiation. Therefore, the compounds contained in chloroform sub-fraction one contains conjugated double bonds. Conjugated double bonds are also found in the phenolic units of habenariol produced by *Habenaria repens* and the lignoids produced *Saururus cernuus* and *Micranthemum umbrosum* (Bolser et al. 1998, Kubanek et al. 2001, Parker et al. 2006, Wilson et al. 1999). Additionally, the flavonoids and C_{18} fatty acid identified as freshwater plant deterrent compounds contain phenyl groups and double bonds, respectively (Parker et al. 2007). Therefore, it is possible that the sub-fraction containing spots that were illuminated by UV radiation is responsible for *S. graminea*'s chemical defenses against *P. clarkia*.

One likely explanation for the loss of deterrent activity in the hexanes-soluble fraction during sub-fraction bioassays is the method of separation. It is common for compounds to irreversibly adsorb to the silica gel column despite flushing with solvents, but this is more likely for polar compounds because silica gel is polar. An additional explanation may have been the

time lag between the hexanes-soluble fraction bioassay and the sub-fractions bioassays. During this time, the active compound(s) may have degraded. However, some of the deterrent compound(s) was still present in the sub-fractions and decreased feeding during the recombined bioassay. In order to avoid problems with loss of activity, the lipophilic Sephadex LH20 column was chosen for the fractionation of the new plant tissue extract. This type of column separates compounds by size rather than polarity, decreasing the likelihood that a compound will stick to the column.

The fresh tissue bioassay that compared constitutive defenses in the stem and leaf portions of *S. graminea* yielded no significant differences (Figure 3a). However, it is surprising that the crayfish consumed equal proportions of the two structures because the stem was bulkier, more buoyant, and more difficult for the crayfish to grasp. Based on these structural inhibitions, it was expected following the first bioassay that the leaf contained more chemical defense than the stem, which made the two portions comparatively edible for the crayfish. The crude extract bioassay was carried out to investigate the presence of higher chemical deterrence in the leaf portion. No significant difference between the consumption of the leaf portion and the stem portion indicate that they have equal magnitudes of chemical defense. Therefore, it can be concluded that the chemical defense of *S. graminea* is distributed throughout the plant.

The future directions of this project include solid phase extraction (SPE) with a reversed phase column to separate the LH-20 sub-fractions 4-6. The remaining LH-20 sub-fractions outside of 3-7 will be tested for deterrence with bioassays. High performance liquid chromatography (HPLC) will help further fractionate the *S. graminea* extract. These fractions will be tested with bioassays and a second round of HPLC may be required. Finally, the deterrent compounds will be identified with NMR, mass spectrometry, infrared spectra, UV, and optical

rotation analysis. Additionally, work has begun to investigate the effects of nutrient excess and limitation on *S. graminea*'s resource allocation to chemical defense. Understanding traits of this defense will help characterize freshwater chemical defenses as a class independent of, and less developed than, marine and terrestrial chemical defenses. Additionally, knowing plant traits that successfully deter *P. clarkii* will be useful for predicting the extent of damage it could cause to an ecosystem as an invasive species.

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